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Vitamin D_3 compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the active form of vitamin D_3, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-$(OH)_2D_3$ mediated apoptosis in MCF-7 breast cancer cells *in vitro*. The effect of 1,25-$(OH)_2D_3$ on MCF-7 cells was compared to that of tumor necrosis factor α (TNFα), which induces apoptosis via a caspase-dependent pathway. Our major findings are that 1,25-$(OH)_2D_3$ induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. Moreover, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-$(OH)_2D_3$ treatment of a MCF-7 cell clone selected for resistance to 1,25-$(OH)_2D_3$ mediated apoptosis. These mitochondrial effects of 1,25-$(OH)_2D_3$ do not require caspase activation, since they are not blocked by the cell permeable caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD.fmk). Although caspase inhibition blocks 1,25-$(OH)_2D_3$ mediated events downstream of mitochondria (such as poly (ADP-ribose) polymerase (PARP) cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-$(OH)_2D_3$ mediated cell death is caspase-independent.

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Carmen J. Marquez 1/28/00

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INTRODUCTION

Vitamin D_3 compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-D_3, the active form of vitamin D_3, acts through the nuclear vitamin D_3 receptor (VDR) and induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. We examined the 1,25-D_3 signaling pathway downstream of the VDR to identify intracellular events involved in 1,25-D_3 mediated apoptosis and to characterize events which are blocked in MCF-7^{D3Res} cells (a vitamin D_3 resistant variant) (Narvaez *et al.*, 1996). In particular, the effects of 1,25-D_3 mediated apoptosis on mitochondrial function and caspase activity were studied and compared to the effects of TNFα. TNFα was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by means of a well-defined pathway triggered by TNFR1, a cell surface death receptor whose signaling results in caspase activation and disruption of mitochondrial function (Budihardjo *et al.*, 1999). Caspases are a family of evolutionarily conserved cysteine proteases that become activated upon proteolytic cleavage, and are responsible for cell disassembly. Cleavage of specific substrates by caspases during apoptosis promotes the degradation of key structural proteins, including PARP, and leads to external display of phosphatidylserine, DNA fragmentation, and cellular condensation (Thornberry and Lazebnik, 1998). Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, release of cytochrome *c*, and production of reactive oxygen species (Green and Reed, 1998; Susin *et al.*, 1998). Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members, including Bax, can trigger these changes. For example, apoptotic signals induce conformational changes in Bax that lead to its translocation to the mitochondria (Tsujimoto and Shimizu, 2000). It may be during this mitochondrial phase that the cell makes a commitment to die. Events downstream of mitochondrial disruption are characterized by the action of caspases and nuclease activators released from mitochondria leading to the ultimate destruction of the cell. While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase dependent pathways. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to determine the involvement of caspase-dependent proteolysis in 1,25-D_3 mediated apoptosis.

RESEARCH ACCOMPLISHMENTS

Previously, in the first Annual Report, I reported data demonstrating that 1,25-D_3 induces apoptosis in MCF-7 cells by disruption of mitochondrial function (a first observation) which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. Moreover, I demonstrated that Bax translocation and mitochondrial disruption do not occur after 1,25-D_3 treatment of the vitamin D_3 resistant variant, MCF-7^{D3Res} cells. These mitochondrial effects of 1,25-D_3 do not require caspase activation, since they are not blocked by the cell permeable caspase inhibitor zVAD.fmk. Although caspase inhibition blocks 1,25-D_3 mediated events downstream of mitochondria (such as PARP cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-D_3 mediated cell death is caspase-independent (submitted manuscript).

To expand on these findings, time course studies of cytochrome *c* release, ROS production, and phosphatidylserine exposure were performed to determine the timing of events that trigger apoptosis. Redistribution of cytochrome *c* from mitochondria to cytosol was detected within 48 hrs of 1,25-D_3

treatment in MCF-7 cells, before any morphological apoptotic features were detected. Long-term exclusion of cytochrome *c* from the electron transport chain can lead to impairment of proton flow and generation of ROS due to incomplete reduction of molecular oxygen. Time course studies have demonstrated that ROS production is enhanced within 72 hrs of 1,25-D_3 treatment in MCF-7 cells. Cytochrome *c* release into the cytosol triggers caspase activity downstream of mitochondria. External display of phosphatidylserine, an early morphological marker of apoptosis, has been shown by others to be provoked by caspases. Annexin V-FITC is a convenient probe for monitoring changes in the distribution of phosphatidylserine in the plasma membrane during apoptosis. The earliest detection of phosphatidylserine exposure by flow cytometry was 96 hrs after the initial treatment with 1,25-D_3 in MCF-7 cells. This demonstrates that mitochondrial disruption occurs prior to activation of downstream caspases.

Time course studies of Bax translocation by immunoblot analysis yielded inconclusive results. Although a time dependent decrease in cytosolic Bax was observed as early as 48 hrs, interpretation of immunoblots of membrane bound Bax which included separate subcellular fractions of non-nuclear (enriched in mitochondria) and nuclear membranes was complicated by the observation that the nuclear fraction was contaminated with mitochondrial proteins. Further studies will be undertaken using immunofluorescent detection with Bax antibody of cells grown and fixed on glass slides.

My observations indicate that 1,25-D_3 triggers both caspase-independent and caspase-dependent pathways in MCF-7 cells, and suggest that 1,25-D_3 can activate downstream effector caspases. Since cytochrome *c* release has been associated with autoactivation of procaspase-9, 1,25-D_3 may activate caspase-dependent pathways via cytochrome *c* release. However, no DEVDase activity was detected in 1,25-D_3 treated cytoplasmic extracts, suggesting that other, possibly unidentified, effector caspases may be activated by 1,25-D_3. Caspase-9 activity assays are currently underway to determine if this caspase is responsible for 1,25-D_3 mediated caspase dependent events. Other caspases will also be examined i.e. caspase-2 and -6.

A role for the pro-apoptotic protein Bax in 1,25-D_3 mediated apoptosis is consistent with previous studies that support a role for Bcl-2, the anti-apoptotic antagonistic partner of Bax, in mediating the effects of 1,25-D_3 on both breast and prostate cancer cells. Thus, 1,25-D_3 down-regulates Bcl-2 (James *et al.*, 1995; Simboli-Campbell *et al.*, 1997) and overexpression of Bcl-2 blocks 1,25-D_3 induced apoptosis (Blutt *et al.*, 2000; Mathiasen *et al.*, 1999). Since Bcl-2 and Bax act antagonistically in the regulation of apoptosis, these data suggest that down-regulation of Bcl-2 in conjunction with translocation of Bax may be necessary for 1,25-D_3 mediated apoptosis. Signals generated by 1,25-D_3 that induce Bax translocation to mitochondria are currently unknown. Since events upstream of Bax translocation to mitochondria in response to 1,25-D_3 are abrogated in the vitamin D_3 resistant MCF-7 variant, comparison of early events in VDR signaling in these cells will be an important subject for future studies.

The original scope of this study was to examine the role phosphorylation plays in vitamin D_3 induced apoptosis. Previous studies have shown that the VDR can be phosphorylated by protein kinase C (PKC) and casein kinase II (Hilliard *et al.*, 1994; Jurutka *et al.*, 1993). Our observation that the phorbol ester TPA, a PKC activator, can potentiate the effects of 1,25-D_3 on induction of apoptosis in breast cancer cells, and more importantly, that TPA can partially sensitize the vitamin D_3 resistant variant to the effects of 1,25-D_3 suggests an important role for phosphorylation in dictating sensitivity to vitamin D_3 mediated apoptosis (Narvaez and Welsh, 1997). Now in addition to looking at phosphorylation, the role of mitochondria and caspase activity in vitamin D_3 medated apoptosis was also examined. I demonstrated that mitochondrial disruption and caspase activity are abrogated in response to 1,25-D_3 treatment in the MCF-7^{D3Res} cells. However, when the MCF-7^{D3Res} cells are treated with 1,25-D_3 in the presence of TPA, activation of caspases is observed as assessed by phosphatidylserine exposure. This indicates that the effects of 1,25-D_3 on mitochondrial disruption and caspase activity

might be sensitized through activators of PKC. By utililizing this vitamin D_3 resistant cell line, we may be able to identify the signals induced by 1,25-D_3 upstream of Bax translocation.

STATEMENT OF WORK

Task 1: *Compare phosphorylation state of the VDR in MCF-7 and MCF-7^{D3res} cells following treatment with ethanol, 1,25-D_3 and TPA. Conduct time couses (up to 24 hrs) and dose responses (1-100 nM).*

Task 2: *Conduct 2D peptide mapping and phosphoamino acid analysis to determine if distinct sites are phosphorylated after treatment with 1,25-D_3 or TPA and determine whether differences correlate to sensitivity to apoptosis.*

Due to technical difficulties as outlined in the first Annual Report, Tasks 1 & 2 were set aside for the time being. However, if TPA alters 1,25-D_3 mediated indices of apoptosis or VDR transactivation (Tasks 3-6), we will work backwards to determine if altered phosphorylation of VDR in MCF-7^{D3Res} cells can be linked to cellular sensitivity to 1,25-D_3 mediated apoptosis.

Task 3: *Monitor biochemical and morphological indices of apoptosis to determine whether differences in phosphorylation patterns correlate to sensitivy to apoptosis.*

This task was expanded to include the effects of 1,25-D_3 on mitochondria and caspase activity. Work on this Task yielded a first observation that 1,25-D_3 induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. In addition, TPA potentiated the effects of 1,25-D_3 on MCF-7^{D3Res} cells by activating downstream caspases, thus indicating that the effects of 1,25-D_3 on mitochondrial disruption and caspase activity might be sensitized through activators of PKC.

Time course of Bax translocation to mitochondria will be conducted using immunocyto fluoresence techniques.

To identify downstream effector caspases of 1,25-D_3 actions, caspase-9 activity assays are currently underway to determine if this caspase is responsible for 1,25-D_3 mediated caspase activation. Other caspases will also be examined i.e. caspase-2 and -6.

Task 4: *Conduct gel shift assays to determine whether phosphorylation affects binding of the VDR to selective VDREs and whether VDR binding is altered in MCF-7 and MCF-7^{D3res} cells after treatment with 1,25-D_3 or TPA ±1,25-D_3.*

Will be completed after Task 6.

Task 5: *Subclone multiple copies of chosen VDREs into Luciferase reporter plasmid.*

We have obtained 24-hydroxylase luciferase promoter construct, which will be used as a representative 1,25-D3 stimulated target gene (see Task 6). Completed.

Task 6: *Conduct transient transfections and Luciferase assays on MCF-7 and MCF-7^{D3Res} cells treated with 1,25-D_3 or TPA ±1,25-D_3 to determine if differences in VDR phosphorylation and sequence specific DNA binding translate to alterations in VDR mediated transactivation in the whole cell system.*

Preliminary studies indicate that basal 24-hydroxylase activity is similar in MCF-7 and MCF-7^{D3Res} cell lines, however, 1,25-D_3 stimulated 24-hydroxylase luciferase activity is blunted in MCF-7^{D3Res} cells compared to parental MCF-7. Effects of TPA pretreatment will be studied next.

KEY RESEARCH ACCOMPLISHMENTS

First Year

- ✓ First observation implicating a role for mitochondrial events in 1,25-D_3 mediated apoptosis.
- ✓ ***Caspase independent events*** involved in 1,25-D_3 mediated apoptosis.
  - Translocation of Bax
  - Release of cytochrome *c*
  - Production of reactive oxygen species
- ✓ ***Caspase dependent events*** involved in 1,25-D_3 mediated apoptosis.
  - PARP cleavage
  - External display of phosphatidylserine
  - DNA fragmentation
- ✓ The observation that 1,25-D_3 mediated cell death is caspase independent.
- ✓ Techniques acquired so far in the course of this study
  - Flow Cytometry
  - Immunoprecipitation
  - Electromobility Shift Assay

Second Year

- ✓ Time course studies of cytochrome *c* release, ROS production, and phosphatidylserine exposure suggests that mitochondrial disruption occurs prior to activation of downstream caspases.

- ✓ The observation that TPA potentiates the effects of 1,25-D_3 on MCF-7^{D3Res} cells by activating downstream caspases, thus indicating that the effects of 1,25-D_3 on mitochondrial disruption and caspase activity might be sensitized through activators of PKC.

- ✓ The observation that 1,25-D_3 stimulated 24-hydroxylase luciferase activity is blunted in MCF-7^{D3Res} cells compared to parental MCF-7.

REPORTABLE OUTCOMES

- ✓ Narvaez CJ and Welsh J (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. Manuscript submitted to a peer-reviewed journal.

- ✓ Narvaez CJ, Waterfall T, Welsh J (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. *Proceedings of the Eleventh Workshop on Vitamin D.* In Press.

- ✓ Abstract promoted to a 10 min presentation at the **Eleventh Workshop on Vitamin D** in Nashville, TN, May 27-June 1, 2000. Abstract Title: "Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells."

- ✓ Poster presented at the ***Era of Hope*** **Department of Defense Breast Cancer Research Progam Meeting** in Atlanta, GA, June 8-11, 2000. Abstract Title: "Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells."

CONCLUSION

1,25-$(OH)_2D_3$ mediates apoptosis of MCF-7 cells through mitochondrial signaling which involves ROS generation, and is regulated by the Bcl-2 family of apoptotic regulators. Caspases act solely as executioners to facilitate 1,25-$(OH)_2D_3$ mediated apoptosis, and caspase activation is not required for induction of cell death by 1,25-$(OH)_2D_3$. Our data suggest distinct differences in the mechanisms of apoptosis induced by 1,25-$(OH)_2D_3$ and TNFα, since inhibition of caspases was able to rescue MCF-7 cells from TNFα, but not 1,25-$(OH)_2D_3$, mediated cell death. Although caspase inhibition blocked biochemical changes associated with caspase activation downstream of mitochondrial perturbations and loss of cytochrome *c*, the commitment of MCF-7 cells to 1,25-$(OH)_2D_3$ mediated apoptosis is clearly caspase-independent.

By understanding the effects of 1,25-D_3 on mitochondria and caspase activity, one can determine how TPA (and PKC pathway) interact with vitamin D signaling in potentiating apoptosis in D_3-resistant MCF-7 cells. This project has important implications for breast cancer since a breast cancer cell that is resistant to the apoptosis-inducing effects of 1,25-D_3 might be sensitized through activators of PKC. This basic knowledge could lead to new therapeutics for treatment of certain forms of breast cancer.

REFERENCES

Blutt, S.E., McDonnell, T.J., Polek, T.C. and Weigel, N.L. (2000) Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2 [see comments]. *Endocrinology*, 141, 10-7.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999) Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol*, 15, 269-90.

Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, 281, 1309-12.

Hilliard, G.M.t., Cook, R.G., Weigel, N.L. and Pike, J.W. (1994) 1,25-dihydroxyvitamin D3 modulates phosphorylation of serine 205 in the human vitamin D receptor: site-directed mutagenesis of this residue promotes alternative phosphorylation. *Biochemistry*, 33, 4300-11.

James, S.Y., Mackay, A.G. and Colston, K.W. (1995) Vitamin D derivatives in combination with 9-cis retinoic acid promote active cell death in breast cancer cells. *J Mol Endocrinol*, 14, 391-4.

Jurutka, P.W., Hsieh, J.C., MacDonald, P.N., Terpening, C.M., Haussler, C.A., Haussler, M.R. and Whitfield, G.K. (1993) Phosphorylation of serine 208 in the human vitamin D receptor. The predominant amino acid phosphorylated by casein kinase II, in vitro, and identification as a significant phosphorylation site in intact cells. *J Biol Chem*, 268, 6791-9.

Mathiasen, I.S., Lademann, U. and Jaattela, M. (1999) Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. *Cancer Res*, 59, 4848-56.

Narvaez, C.J., Vanweelden, K., Byrne, I. and Welsh, J. (1996) Characterization of a vitamin D3-resistant MCF-7 cell line. *Endocrinology*, 137, 400-9.

Narvaez, C.J. and Welsh, J. (1997) Differential effects of 1,25-dihydroxyvitamin D3 and tetradecanoylphorbol acetate on cell cycle and apoptosis of MCF-7 cells and a vitamin D3-resistant variant. *Endocrinology*, 138, 4690-8.

Simboli-Campbell, M., Narvaez, C.J., van Weelden, K., Tenniswood, M. and Welsh, J. (1997) Comparative effects of 1,25(OH)2D3 and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells. *Breast Cancer Res Treat*, 42, 31-41.

Susin, S.A., Zamzami, N. and Kroemer, G. (1998) Mitochondria as regulators of apoptosis: doubt no more. *Biochim Biophys Acta*, 1366, 151-65.

Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science*, 281, 1312-6.

Tsujimoto, Y. and Shimizu, S. (2000) Bcl-2 family: life-or-death switch. *FEBS Lett*, 466, 6-10.

ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS OF MCF-7 BREAST CANCER CELLS

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Running Title: Caspase-independent apoptosis mediated by vitamin D_3

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SUMMARY

Vitamin D_3 compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the active form of vitamin D_3, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-$(OH)_2D_3$ mediated apoptosis in MCF-7 breast cancer cells *in vitro*. The effect of 1,25-$(OH)_2D_3$ on MCF-7 cells was compared to that of tumor necrosis factor α (TNFα), which induces apoptosis via a caspase-dependent pathway. Our major findings are that 1,25-$(OH)_2D_3$ induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. Moreover, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-$(OH)_2D_3$ treatment of a MCF-7 cell clone selected for resistance to 1,25-$(OH)_2D_3$ mediated apoptosis. These mitochondrial effects of 1,25-$(OH)_2D_3$ do not require caspase activation, since they are not blocked by the cell permeable caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD.fmk). Although caspase inhibition blocks 1,25-$(OH)_2D_3$ mediated events downstream of mitochondria (such as poly (ADP-ribose) polymerase (PARP) cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-$(OH)_2D_3$ mediated cell death is caspase-independent.

INTRODUCTION

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the active form of vitamin D_3, acts through the nuclear vitamin D_3 receptor (VDR) and is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo* (1). A variety of synthetic vitamin D_3 analogs that induce mammary tumor regression in animals are now undergoing clinical trials in human patients (2,3). Our lab has shown that 1,25-$(OH)_2D_3$ induces morphological and biochemical markers of apoptosis (chromatin and nuclear matrix condensation, and DNA fragmentation) in MCF-7 breast cancer cells (4,5), however, the precise mechanism by which 1,25-$(OH)_2D_3$ and the VDR mediate apoptosis is poorly understood.

To characterize the mechanisms of 1,25-$(OH)_2D_3$ mediated apoptosis in breast cancer cells, we compared specific intracellular events in MCF-7 cells after treatment with 1,25-$(OH)_2D_3$ or TNFα. TNFα was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by a well-defined pathway triggered by TNFR1, a cell surface death receptor. Death receptors contain homologous cytoplasmic regions termed "death domains" which transmit apoptotic signals through recruitment of adaptor molecules that activate caspases, a family of cysteine proteases involved in cell disassembly. The best-characterized death receptors (Fas, TNFR1) use FADD and TRADD adaptors to recruit and activate caspase-8 (6). Cleavage of specific substrates by caspases during apoptosis promotes the degradation of key structural proteins, including poly (ADP-ribose) polymerase (PARP), and lead to external display of phosphatidylserine (PS), DNA fragmentation and cellular condensation (7).

Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, release of cytochrome *c* and apoptosis-inducing factor (AIF), and production of reactive oxygen species (ROS) (8,9). Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members, including Bax, can trigger these changes. For example, apoptotic signals induce conformational changes in Bax, which lead to exposure of the pro-apoptotic BH3 domain, and translocation to the mitochondria (10). The effects of pro-apoptotic Bcl-2 family members are achieved by both caspase-dependent and caspase-independent mechanisms (11,12).

While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase-dependent pathways. To probe the mechanisms whereby vitamin D_3 signaling modulates apoptosis in MCF-7 cells, we studied the effects of 1,25-$(OH)_2D_3$ on mitochondrial function and caspase activity using a cell permeable inhibitor of caspase-related proteases (zVAD.fmk). In addition, the effects of 1,25-$(OH)_2D_3$ and TNFα on a vitamin D_3 resistant variant of MCF-7 cells (MCF-7^{D3Res} cells) were examined to identify events that contribute to vitamin D_3 resistance (13,14). The MCF-7^{D3Res} cells do not undergo cell cycle arrest or apoptosis in response to 1,25-$(OH)_2D_3$, however, these cells retain sensitivity to other inducers of apoptosis such as TNFα and antiestrogens (13).

Our major findings are that 1,25-$(OH)_2D_3$ induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of ROS. These mitochondrial effects of 1,25-$(OH)_2D_3$ do not require caspase activation, since they are not blocked by the caspase inhibitor zVAD.fmk. Although caspase inhibition blocks 1,25-$(OH)_2D_3$ mediated events downstream of mitochondria (such as PARP cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-$(OH)_2D_3$ mediated cell death is caspase-independent.

EXPERIMENTAL PROCEDURES

Cells and cell culture

MCF-7 cells (originally obtained from ATCC) were used to generate the vitamin D_3 resistant variant (MCF-7^{D3Res}) that has been previously described (13). Both cell lines were cultured in α-MEM medium (Life Technologies, Inc., Gaithersburg, MD) containing 25 mM HEPES and 5% FBS (Life Technologies). Cells were routinely plated at 5000 cells/cm^2 and passaged every 3-4

days. Stock cultures of MCF-7^{D3Res} cells were routinely grown in medium containing 100 nM 1,25-$(OH)_2D_3$ (kindly provided by LEO Pharmaceuticals, Ballerup, Denmark). For experiments, MCF-7 and MCF-7^{D3Res} cells were plated in α-MEM containing 5% FBS plus antibiotics, and treated with 1,25-$(OH)_2D_3$ or ethanol vehicle 2 days after plating. Parallel cultures were treated with TNFα (Sigma, St Louis, MO) 2-3 days before scheduled harvest of 1,25-$(OH)_2D_3$ treated dishes. Caspase inhibitors, zVAD.fmk or zDEVD.fmk (Enzyme Systems Products, Livermore, CA) were added at the same time (1,25-$(OH)_2D_3$ or TNFα) or two days after (1,25-$(OH)_2D_3$) initial treatment. For cell growth assays, cells were seeded at 1000 cells/well in 24-well plates, treated for the indicated times and analyzed by crystal violet assay. Briefly, cells were fixed with 1% glutaraldehyde for 15 min, incubated with 0.1% crystal violet (Fisher Scientific, Pittsburgh, PA) for 30 min, destained with H_2O and solubilized with 0.2% Triton X-100. Absorbance at 562 nm (minus background at 630 nm) was determined on a microtiter plate reader.

Clonogenicity Assay

MCF-7 cells were incubated with 1,25-$(OH)_2D_3$ for 6 days or TNFα for 1 day in the presence or absence of 25 μM zVAD.fmk. For 1,25-$(OH)_2D_3$ treatment, medium was replaced every two days. After treatments, cells were trypsinized, media and washes were pooled, cells were pelleted by centrifugation, and resuspended in fresh medium. The cells were seeded in 96-well plates at 5, 50, 500, and 2500 cells/well in 24 replicates. After 14 days, the cells were fixed and stained with crystal violet as described above, and clonogenic potential was estimated by counting positive wells (15).

Subcellular Fractionation

Cells were trypsinized, pooled together with media and washes containing floating cells, and pelleted by centrifugation at 500xg for 3 min at 4°C. Pellets were resuspended with 3 volumes of Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 1 mM DTT, 250 mM sucrose plus protease and phosphatase inhibitors), lysed with a Dounce homogenizer, and fractionated by differential centrifugation (16). Briefly, homogenates were centrifuged twice at 500xg for 5 min at 4°C, and the nuclear pellet was resuspended in Buffer A, sonicated 2x10 s, and stored at -80°C in multiple aliquots. The supernatants were combined and further centrifuged at 10,000xg for 30 min at 4°C, and the resultant mitochondrial pellets were resuspended in Buffer A, sonicated, and stored at -80°C in multiple aliquots. The supernatant from the 10,000xg spin was further centrifuged at 100,000xg for 1 h at 4°C. The resulting supernatant was designated S100 (containing cytosol) and stored at -80°C in multiple aliquots. Protein concentrations were determined by the Micro BCA protein assay (Pierce, Rockford, IL).

Immunoblot analysis

Subcellular fractions isolated as described above were solubilized in Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. Proteins derived from mitochondria and/or S100 extracts were immunoblotted with Bax rabbit polyclonal (13666E; PharMingen, San Diego, CA) or cytochrome *c* mouse monoclonal antibodies (7H8.2C12; PharMingen) diluted 1:500 or 1:250, respectively, in PBS/5% skim milk. S100 extracts were also probed with cytochrome oxidase subunit II mouse monoclonal antibody (clone 12C4-F12, Molecular Probes, Eugene, OR) to exclude mitochondrial contamination. Nuclear extracts were probed with PARP mouse monoclonal antibody (C2.10, Enzyme Systems Products) diluted 1:1000. Specific antibody binding was detected by horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:5000 in PBS/5% skim milk/0.1% Tween 20 and autoradiography with enhanced chemiluminescence (Pierce).

Immunocytochemistry

MCF-7 cells grown on Lab-Tek II chamber slides (Fisher Scientific) were treated with ethanol vehicle, 100 nM 1,25-$(OH)_2D_3$, or 2.5 ng/ml TNFα for 96 h (ethanol, 1,25-$(OH)_2D_3$) or 48 h (TNFα) in the presence or absence of 25 μM zVAD.fmk. The cells were fixed in 4% formaldehyde in PBS for 5 min at room temperature, permeabilized in methanol at -20°C for 5 min, and blocked overnight with PBS/1% BSA containing 0.02% sodium azide. The slides were then incubated with cytochrome *c* mouse monoclonal antibody (6H2.B4; PharMingen), diluted 1:100 in blocking buffer, for 3 h at 37°C in a humidified chamber. Slides were washed 3x5 min with PBS followed by incubation for 1 h at room temperature with anti-mouse secondary antibody conjugated to ALEXA-488 (a photostable dye with spectral properties similar to fluorescein; Molecular Probes) diluted 1:50 in blocking buffer. Slides were washed 3x5 min with PBS, incubated for 15 min at room temperature with 1 μg/ml Hoechst 33258 (Sigma), washed 5x5 min with PBS, rinsed with dH_20, and coverslips were applied with an antifade reagent. Fluorescence was detected using Olympus AX70 microscope equipped with a Spot RT digital camera.

Flow cytometry

For analysis of mitochondrial membrane potential and reactive oxygen species, cells harvested by trypsinization were pooled with media plus washes and pelleted by centrifugation. Cell suspensions ($1x10^6$ cells) were incubated with 1 μM tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) in PBS containing 130 mM KCl to abolish the plasma membrane potential. After incubation for 10 min at 37°C, cells were washed once in PBS and then analysed for TMRE red fluorescence by flow cytometry. Live cells rapidly and reversibly take up TMRE, and accumulation of the dye in mitochondria has been shown to be potential driven (17). For analysis of ROS, cell suspensions ($5x10^5$ cells) were incubated with 4 μM hydroethidine (HE, Molecular Probes) in PBS for 15 min at 37°C, and conversion of HE to ethidium by superoxide anion was analyzed by flow cytometry.

For analysis of DNA fragmentation and phosphatidylserine externalization, MCF-7 cells were harvested by trypsinization, collected by centrifugation, fixed in 2% formaldehyde in PBS, and permeabilized in 70% EtOH at -20°C. DNA strand breaks in cells undergoing apoptosis were indirectly labeled with bromodeoxyuridine (Br-dUTP) by terminal transferase (Boehringer Mannheim,

Indianapolis, IN) and detected by FITC-conjugated monoclonal antibody to Br-dUTP using the APO-BRDU kit according to manufacturer's protocol (Phoenix Flow Systems, San Diego, CA). Cells were counterstained with 5 μg/ml propidium iodide (PI; Sigma) containing RNAse A (Boehringer Mannheim) for detection of total DNA, and two color analysis of DNA strand breaks and cell cycle was achieved by flow cytometry.

For detection of phosphatidylserine (PS) externalization, $1x10^6$ cells were incubated in the presence of 10 μg/ml annexin V-FITC (BioWhittaker, Walkersville, MD) and 5 μg/ml PI in binding buffer (10 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 2.5 mM $CaCl_2$) for 15 min at 37°C. Cells were washed twice in binding buffer, fixed in 2% formaldehyde in PBS for 15 min on ice, and then washed two more times in PBS/0.2% BSA. Pellets were resuspended in PBS/0.2% BSA and analyzed by flow cytometry. There were less than 1% PI^+ cells in the population and were therefore excluded from analysis.

All flow cytometric analyses were performed on an Epics XL Flow Cytometer (Coulter Corp, Miami, FL) equipped with an argon laser. TMRE and HE were analyzed on FL3 using a 620 nm band pass filter. For DNA fragmentation analysis, FITC was analyzed on FL1 using a 520 nm band pass filter and PI was analyzed on FL3 with no color compensation. For PS externalization, annexin V-FITC was analyzed on FL1 and PI was analyzed on FL2 (580 nm band pass filter) using software color compensation. Data was modeled with the MULTIPLUS AV software (Phoenix Flow Systems).

Caspase Activity Assay

Caspase activity was analyzed with the ApoAlert CPP32/Caspase-3 assay kit according to manufacturer's protocol (CLONTECH, Palo Alto, CA). Briefly, after harvesting by trypsinization, $2x10^6$ cells were pelleted and stored at -20°C. For analysis, cell pellets were lysed, re-pelleted to remove cell debris, and supernatants were incubated with 50 μM DEVD-AFC for 1 h at 37°C. The samples were analyzed using a fluorescence spectrophotometer with Ex=380 nm and Em=508 nm.

Statistical Evaluation

Data are expressed as mean ± SEM. One-way ANOVA was used to assess statistical significance between means. Differences between means were considered significant if p values less than 0.05 were obtained with the Bonferroni method using GraphPad Instat software (GraphPad Software, San Diego, CA).

RESULTS

Disruption of mitochondrial function, as determined by subcellular localization of Bax and cytochrome *c*, and ROS generation, by 1,25-$(OH)_2D_3$.

To identify specific intracellular events involved in 1,25-$(OH)_2D_3$ mediated apoptosis, we examined the signaling pathway downstream of the VDR in MCF-7 cells. Since disruption of mitochondrial function is a primary event in apoptosis that can be triggered by translocation of Bax to mitochondrial outer membrane, we first examined the subcellular distribution of Bax after 1,25-$(OH)_2D_3$ treatment of MCF-7 cells. As demonstrated in Fig. 1, Bax redistribution from the cytosolic to the mitochondrial fraction occurred after treatment with 1,25-$(OH)_2D_3$ or TNFα in MCF-7 cells (Fig. 1, *Top*). Not only was Bax translocated to mitochondria, but both 1,25-$(OH)_2D_3$ and TNFα treated cells exhibited cleavage of Bax from the intact 21 kDa protein to an 18 kDa fragment, an observation which is consistent with reports of Bax cleavage during drug-induced apoptosis (18). In both 1,25-$(OH)_2D_3$ and TNFα treated cells, the Bax cleavage product was detected in mitochondrial, but not cytosolic, fractions, and others have proposed that Bax cleavage enhances homodimerization and its pro-apoptotic properties (19). These are the first data to implicate translocation and cleavage of Bax during 1,25-$(OH)_2D_3$ induced apoptosis. To determine the relationship between Bax translocation and sensitivity to 1,25-$(OH)_2D_3$ induced apoptosis, we examined the subcellular distribution of Bax in a vitamin D_3 resistant variant of MCF-7 cells which does not undergo apoptosis after treatment with 1,25-$(OH)_2D_3$ but retains sensitivity to other triggers, including TNFα (Narvaez and Welsh, unpublished data). In the MCF-7^{D3Res} cells, 1,25-$(OH)_2D_3$ did not induce translocation or cleavage of Bax (Fig. 1, *Bottom*). However, in these cells, Bax translocation and cleavage was triggered by TNFα, indicating that Bax functions appropriately in MCF-7^{D3Res} cells during apoptosis induced by agents other than 1,25-$(OH)_2D_3$. The inability of Bax to redistribute to mitochondria in response to 1,25-$(OH)_2D_3$ in the vitamin D_3 resistant variant suggests that Bax translocation may be a critical initiating event in 1,25-$(OH)_2D_3$ mediated apoptosis of MCF-7 cells.

Translocation of Bax to mitochondria has been associated with release of cytochrome *c*, an event that is considered a commitment point for activation of apoptosis. As expected for viable cultures, no cytochrome *c* was detected in cytosolic fractions from MCF-7 cells treated with ethanol vehicle for up to 120 h (Fig. 2A). In contrast, redistribution of cytochrome *c* from mitochondria to cytosol was detected within 48 h of 1,25-$(OH)_2D_3$ treatment in MCF-7 cells, before any morphological apoptotic features were detected. The absence of cytochrome oxidase in cytosolic fractions confirmed that extracts were free of mitochondrial contamination (data not shown). In MCF-7^{D3Res} cells, 1,25-$(OH)_2D_3$ did not trigger release of cytochrome *c*, however, cytochrome *c* was detected in cytosolic fractions after TNFα treatment of both MCF-7 and MCF-7^{D3Res} cell lines (Fig. 2B, see also Fig. 7).

Long-term exclusion of cytochrome *c* from the electron transport chain can lead to impairment of proton flow and generation of ROS due to incomplete reduction of molecular oxygen. Hence, mitochondrial generation of ROS in response to 1,25-$(OH)_2D_3$ and TNFα was examined by flow cytometry. Production of superoxide anion was indirectly assessed as oxidation of hydroethidine to ethidium, which fluoresces red upon DNA intercalation. As presented in Fig. 3, ROS production was enhanced by 1,25-$(OH)_2D_3$ in

MCF-7, but not MCF-7^{D3Res}cells, whereas TNFα increased ROS production comparably in both cell lines. Time course studies have demonstrated that ROS production is enhanced within 72 h of 1,25-$(OH)_2D_3$ treatment in MCF-7 cells (data not shown).

1,25-$(OH)_2D_3$ mediates PARP cleavage, PS externalization, and DNA fragmentation in a caspase-dependent manner.

Cytochrome *c* released into the cytosol is thought to trigger caspase activation downstream of mitochondria through binding to Apaf-1 and autoactivation of procaspase-9. Activated caspase-9 can activate additional effector caspases responsible for cell disassembly and events such as PS externalization, PARP cleavage and DNA fragmentation. To determine the involvement of caspase-dependent proteolysis in 1,25-$(OH)_2D_3$ mediated apoptosis, we examined whether a broad-spectrum cell permeable caspase inhibitor (zVAD.fmk) could abrogate the effects of 1,25-$(OH)_2D_3$ in MCF-7 cells.

Proteolytic activity associated with caspase activation was analyzed by three distinct methods: cleavage of an endogenous caspase substrate (PARP), and flow cytometric analysis of PS exposure and DNA fragmentation, which others have shown are provoked by caspases (7). As demonstrated in Fig. 4A, PARP was cleaved after treatment of MCF-7 cells with either 1,25-$(OH)_2D_3$ or TNFα, and in both cases, cleavage was blocked by zVAD.fmk. Furthermore, both 1,25-$(OH)_2D_3$ and TNFα induced PS externalization, which was also completely blocked by zVAD.fmk (Fig. 4B). Finally, the effects of 1,25-$(OH)_2D_3$ and TNFα on DNA fragmentation was assessed as terminal transferase mediated incorporation of bromodeoxyuridine, detected by FITC-conjugated anti-bromodeoxyuridine antibody by flow cytometry (Fig. 5). 1,25-$(OH)_2D_3$ treatment of MCF-7 cells induced DNA fragmentation primarily in the G_1 phase of the cell cycle, with only a small population of cells (5%) accumulating in sub-G_1. TNFα treatment induced extensive DNA fragmentation in the G_1 phase of the cell cycle, with accumulation of 29% of the population in sub-G_1. Despite the differences in the magnitude and profiles of DNA fragmentation between 1,25-$(OH)_2D_3$ and TNFα treated cells, zVAD.fmk completely blocked DNA fragmentation induced by both agents.

Caspase activity induced by 1,25-$(OH)_2D_3$ is not DEVDase.

DEVDase cleavage activity was measured with the fluorogenic substrate DEVD-AFC, which can detect activity of caspases-3 and -7. Since MCF-7 cells do not express functional caspase-3 (data not shown, ref. (20)), any DEVDase activity detected in these cells would most likely correspond to caspase-7. For these experiments, MCF-7 cells were pretreated with 1,25-$(OH)_2D_3$ for 2 days before cytosolic extracts were collected for DEVDase activity assays at the indicated time points. As demonstrated in Fig. 6, TNFα, but not 1,25-$(OH)_2D_3$, induced DEVDase cleavage activity in MCF-7 cells. Even with extended treatment times in additional studies, no DEVDase cleavage activity could be detected after 1,25-$(OH)_2D_3$ treatment of MCF-7 cells (data not shown). This observation indicates that other known or as yet unidentified effector caspases may be activated by 1,25-$(OH)_2D_3$, which mediate PARP cleavage, PS exposure, or DNA fragmentation.

Caspase inhibition does not block 1,25-$(OH)_2D_3$ mediated cytochrome *c* release, mitochondrial dysfunction or cell death.

Since zVAD.fmk blocked 1,25-$(OH)_2D_3$ mediated caspase-dependent events downstream of mitochondria, we examined the effects of the caspase inhibitor on cytochrome *c* release and mitochondrial function. As shown in Fig. 7, zVAD.fmk did not abrogate 1,25-$(OH)_2D_3$ mediated cytochrome *c* release or ROS production under the same conditions where PS exposure, PARP cleavage and DNA fragmentation were blocked. In contrast, the caspase inhibitor effectively blocked cytochrome *c* release and ROS generation triggered by TNFα (Fig. 7A, 7C).

To further probe mitochondrial function, the membrane potential sensitive probe TMRE was used to detect mitochondrial membrane potential by flow cytometry. 1,25-$(OH)_2D_3$ treatment significantly enhanced the percentage of cells with reduced mitochondrial membrane potential, and zVAD.fmk did not block the decrease in mitochondrial membrane potential induced by 1,25-$(OH)_2D_3$. TNFα treatment also enhanced the percentage of cells with decreased mitochondrial membrane potential, however, in contrast to 1,25-$(OH)_2D_3$, the effect of TNFα was completely blocked by zVAD.fmk (Fig. 7B).

Subcellular localization of cytochrome *c* protein was examined by fluorescence microscopy to confirm the finding that cytochrome *c* release can proceed independently of caspase activation after 1,25-$(OH)_2D_3$ treatment. In Fig. 8, cytochrome *c* fluorescence (middle panels) is presented alongside phase contrast (top panels) and Hoechst nuclear staining (bottom panels) to compare cytochrome *c* localization in individual viable and apoptotic cells. In vehicle treated control cells, apoptotic morphology was not present, and cytochrome *c* staining was restricted to punctate cytoplasmic regions, consistent with mitochondrial localization (Fig. 8). After treatment with 1,25-$(OH)_2D_3$ or TNFα, apoptotic cells identified by phase contrast and Hoechst nuclear staining exhibited chromatin condensation, nuclear fragmentation, and cytosolic vacuolization. In these apoptotic cells, diffuse cytoplasmic cytochrome *c* staining was detected throughout the cell, which obscured the nuclei, consistent with redistribution of cytochrome *c* from mitochondria to cytoplasm (21). Consistent with the immunoblotting data (Fig. 7A), treatment with zVAD.fmk failed to prevent 1,25-$(OH)_2D_3$ mediated cytochrome *c* release, as demonstrated by persistence of diffuse cytoplasmic cytochrome *c* staining in 1,25-$(OH)_2D_3$ plus zVAD.fmk treated cells. However, zVAD.fmk did prevent the morphological signs of apoptosis, including chromatin condensation and nuclear fragmentation, consistent with its ability to block PS redistribution, PARP cleavage, and DNA fragmentation induced by 1,25-$(OH)_2D_3$.

Since zVAD.fmk did not block cytochrome *c* release or mitochondrial dysfunction induced by 1,25-$(OH)_2D_3$, but did protect MCF-7 cells from morphological signs of apoptosis, including DNA fragmentation, we examined whether zVAD.fmk treated cells actually remained viable and/or maintained clonogenic potential. As shown in Fig. 9, both zVAD.fmk and zDEVD.fmk caspase inhibitors rescued MCF-7 cells from TNFα mediated cell death, as demonstrated by total cell numbers, with zVAD.fmk offering the greater protection. However, neither zVAD.fmk nor zDEVD.fmk caspase inhibitors could protect MCF-7 cells from 1,25-$(OH)_2D_3$

mediated apoptosis, since the reduction in total cell number was not abrogated by either inhibitor (Fig. 9). Finally, the clonogenic potential was determined after treatment of cells with 1,25-$(OH)_2D_3$ or TNFα in the presence or absence of zVAD.fmk followed by re-plating at limiting dilutions in fresh medium. In vehicle control treated cultures, at least 1 out of 5 cells had the ability to produce clones. In TNFα treated cultures, clonogenicity was less than 1 out of 500 cells (f<0.002) but in the presence of zVAD.fmk, the frequency of cells with clonogenic potential was significantly increased ($f \geq 0.2$). In 1,25-$(OH)_2D_3$ treated cultures, clonogenicity was less than 1 out of 50 cells (f <0.02), and this was not enhanced in the presence of zVAD.fmk.

DISCUSSION

Here we report for the first time that 1,25-$(OH)_2D_3$ induces apoptosis in MCF-7 cells by disruption of mitochondrial function, which is accomplished by translocation of Bax to mitochondria, and increased permeability of the outer mitochondrial membrane. Of particular interest, neither Bax translocation nor the mitochondrial disruption is induced by 1,25-$(OH)_2D_3$ in a variant line of MCF-7 cells selected for resistance to 1,25-$(OH)_2D_3$ mediated apoptosis (13). Collectively, these data implicate an essential role for mitochondrial signaling in the induction of apoptosis by 1,25-$(OH)_2D_3$ and identify the pro-apoptotic protein Bax as an important downstream target of the VDR in MCF-7 cells.

In addition to Bax translocation, we report that treatment of MCF-7 cells with 1,25-$(OH)_2D_3$ induces cytochrome c release and ROS generation, events which have been observed in cells induced to undergo apoptosis by overexpression of Bax (22,23). These data further support the concept that 1,25-$(OH)_2D_3$ mediated apoptosis may be driven by Bax translocation. A role for the pro-apoptotic protein Bax in 1,25-$(OH)_2D_3$ mediated apoptosis is consistent with previous studies that support a role for Bcl-2, the anti-apoptotic antagonistic partner of Bax, in mediating the effects of 1,25-$(OH)_2D_3$ on both breast and prostate cancer cells. Thus, 1,25-$(OH)_2D_3$ down-regulates Bcl-2 (24,25) and overexpression of Bcl-2 blocks 1,25-$(OH)_2D_3$ induced apoptosis (26,27). Since Bcl-2 and Bax act antagonistically in the regulation of apoptosis, these data suggest that down-regulation of Bcl-2 in conjunction with translocation of Bax may be necessary for 1,25-$(OH)_2D_3$ mediated apoptosis. Further studies will be necessary to identify the signals generated by 1,25-$(OH)_2D_3$ that induce Bax translocation to mitochondria. Since events upstream of Bax translocation to mitochondria in response to 1,25-$(OH)_2D_3$ are abrogated in the vitamin D_3 resistant MCF-7 variant, comparison of early events in VDR signaling in these cells will be an important subject for future studies.

Examination of events downstream of mitochondria indicated that 1,25-$(OH)_2D_3$ induced features of apoptosis associated with caspase activation, such as PARP cleavage, PS exposure and DNA fragmentation. To determine whether caspase activation was required for 1,25-$(OH)_2D_3$ mediated apoptosis, we used the broad spectrum, cell permeable caspase inhibitor zVAD.fmk. We observed that 1,25-$(OH)_2D_3$ signaling on mitochondria does not require caspase activation, since zVAD.fmk was unable to block 1,25-$(OH)_2D_3$ induced cytochrome c release, decrease in mitochondrial membrane potential, or ROS production. Again, this is consistent with apoptosis driven by Bax translocation, which promotes cytochrome c release via caspase-independent pathways (28-31). Our data also complement that of Mathiasen *et al* (27) who reported that inhibition of caspase activity by overexpression of CrmA, a cowpox-derived caspase inhibitor, or caspase inhibitory peptides (Ac-DEVD-CHO, Ac-IETD-CHO, and zVAD.fmk) did not block vitamin D_3 mediated growth arrest or apoptosis.

Although caspase inhibition did not block mitochondrial events induced by 1,25-$(OH)_2D_3$, zVAD.fmk did block events downstream of mitochondria such as PARP cleavage, external display of PS, and DNA fragmentation. These findings are similar to reports of Bax induced apoptosis, where caspase inhibitors had no effect on Bax-induced cytochrome c release or mitochondrial disruption, but prevented cleavage of nuclear and cytosolic substrates and DNA degradation (28-31). However, our data conflict with that of Mathiasen *et al*, who observed that zVAD.fmk did not block 1,25-$(OH)_2D_3$ mediated DNA fragmentation in MCF-7 cells (27). This discrepancy may reflect differences in doses (1 μM vs. 25 μM) or experimental design between the two studies. The lower dose of zVAD.fmk (1 μM) used by Mathiasen *et al* may have been insufficient to block mitochondrial-initiated caspases (caspase-9) (32).

The data presented in this paper indicates that 1,25-$(OH)_2D_3$ triggers both caspase-independent and caspase-dependent pathways in MCF-7 cells, and suggests that 1,25-$(OH)_2D_3$ can activate downstream effector caspases. Since cytochrome c release has been associated with autoactivation of procaspase-9, 1,25-$(OH)_2D_3$ may activate caspase-dependent pathways via cytochrome c release. However, no DEVDase activity was detected in 1,25-$(OH)_2D_3$ treated cytoplasmic extracts, suggesting that other, possibly unidentified, effector caspases may be activated by 1,25-$(OH)_2D_3$, or that caspase-dependent events occur at later stages in the apoptotic program. Although blocking caspase activation prevented some of the morphological aspects of 1,25-$(OH)_2D_3$ mediated apoptosis, MCF-7 cells were not rescued from death by zVAD.fmk. This finding is consistent with reports that many cell types eventually die by a slower, non-apoptotic cell death if caspases are inactivated (8). These data support the concept that mitochondrial damage represents a cell death commitment step in the course of apoptosis induced by many stimuli (33), including 1,25-$(OH)_2D_3$.

In summary, 1,25-$(OH)_2D_3$ mediates apoptosis of MCF-7 cells through mitochondrial signaling which involves ROS generation, and is regulated by the Bcl-2 family of apoptotic regulators. Caspases act solely as executioners to facilitate 1,25-$(OH)_2D_3$ mediated apoptosis, and caspase activation is not required for induction of cell death by 1,25-$(OH)_2D_3$. Our data suggest distinct differences in the mechanisms of apoptosis induced by 1,25-$(OH)_2D_3$ and TNFα, since inhibition of caspases was able to rescue MCF-7 cells from TNFα, but not 1,25-$(OH)_2D_3$, mediated cell death. Although caspase inhibition blocked biochemical changes associated with caspase activation downstream of mitochondrial perturbations and loss of cytochrome c, the commitment of MCF-7 cells to 1,25-$(OH)_2D_3$ mediated apoptosis is clearly caspase-independent.

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REFERENCES

1. VanWeelden, K., Flanagan, L., Binderup, L., Tenniswood, M., and Welsh, J. (1998) *Endocrinology* **139,** 2102-2110
2. Gulliford, T., English, J., Colston, K. W., Menday, P., Moller, S., and Coombes, R. C. (1998) *Br J Cancer* **78,** 6-13
3. Colston, K. W., Mackay, A. G., James, S. Y., Binderup, L., Chander, S., and Coombes, R. C. (1992) *Biochem Pharmacol* **44,** 2273-2280
4. Simboli-Campbell, M., Narvaez, C. J., Tenniswood, M., and Welsh, J. (1996) *J Steroid Biochem Mol Biol* **58,** 367-376
5. Welsh, J., VanWeelden, K., Flanagan, L., Byrne, I., Nolan, E., and Narvaez, C. J. (1998) *Subcell Biochem* **30,** 245-270
6. Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) *Annu Rev Cell Dev Biol* **15,** 269-290
7. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312-1316
8. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309-1312
9. Susin, S. A., Zamzami, N., and Kroemer, G. (1998) *Biochim Biophys Acta* **1366,** 151-165
10. Tsujimoto, Y., and Shimizu, S. (2000) *FEBS Lett* **466,** 6-10
11. Fitch, M. E., Chang, C. M., and Parslow, T. G. (2000) *Cell Death Differ* **7,** 338-349
12. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) *J Cell Biol* **144,** 891-901
13. Narvaez, C. J., Vanweelden, K., Byrne, I., and Welsh, J. (1996) *Endocrinology* **137,** 400-409
14. Narvaez, C. J., and Welsh, J. (1997) *Endocrinology* **138,** 4690-4698
15. Amarante-Mendes, G. P., Finucane, D. M., Martin, S. J., Cotter, T. G., Salvesen, G. S., and Green, D. R. (1998) *Cell Death Differ* **5,** 298-306
16. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997) *Science* **275,** 1129-1132
17. Ehrenberg, B., Montana, V., Wei, M., Wuskell, J., and Loew, L. (1988) *Biophys. J.* **53,** 785-794
18. Wood, D. E., Thomas, A., Devi, L. A., Berman, Y., Beavis, R. C., Reed, J. C., and Newcomb, E. W. (1998) *Oncogene* **17,** 1069-1078
19. Wood, D. E., and Newcomb, E. W. (2000) *Exp Cell Res* **256,** 375-382
20. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) *J Biol Chem* **273,** 9357-9360
21. Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., and Borner, C. (1998) *Nature* **391,** 496-499
22. Xiang, J., Chao, D. T., and Korsmeyer, S. J. (1996) *Proc Natl Acad Sci U S A* **93,** 14559-14563
23. Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G., and Green, D. R. (1999) *J Biol Chem* **274,** 2225-2233
24. James, S. Y., Mackay, A. G., and Colston, K. W. (1995) *J Mol Endocrinol* **14,** 391-394
25. Simboli-Campbell, M., Narvaez, C. J., van Weelden, K., Tenniswood, M., and Welsh, J. (1997) *Breast Cancer Res Treat* **42,** 31-41
26. Blutt, S. E., McDonnell, T. J., Polek, T. C., and Weigel, N. L. (2000) *Endocrinology* **141,** 10-17
27. Mathiasen, I. S., Lademann, U., and Jaattela, M. (1999) *Cancer Res* **59,** 4848-4856
28. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) *EMBO J* **17,** 3878-3885
29. Hsu, Y. T., Wolter, K. G., and Youle, R. J. (1997) *Proc Natl Acad Sci U S A* **94,** 3668-3672
30. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998) *Proc Natl Acad Sci U S A* **95,** 4997-5002
31. Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J., and Shore, G. C. (1998) *J Cell Biol* **143,** 207-215
32. Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M. (1998) *J Exp Med* **187,** 587-600
33. Miyashita, T., Nagao, K., Krajewski, S., Salvesen, G. S., Reed, J. C., Inoue, T., and Yamada, M. (1998) *Cell Death Differ* **5,** 1034-1041

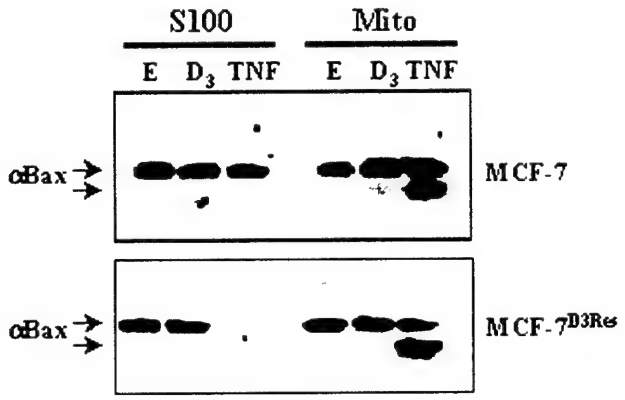

FIG. 1. Subcellular distribution of Bax after treatment with 1,25-$(OH)_2D_3$ or TNFα in parental MCF-7 or MCF-7^{D3Res} cells. MCF-7 or MCF-7^{D3Res} cells were plated at a density of $2x10^5$ cells/150-mm dish. Two days after plating, cells were treated with vehicle control (ethanol) or 100 nM 1,25-$(OH)_2D_3$ for 96 h, or with 2.5 ng/ml TNFα for 48 h. Mitochondria and S100 isolated as described in *Experimental Procedures* were separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal antibody to Bax. The results are representative of at least three independent experiments.

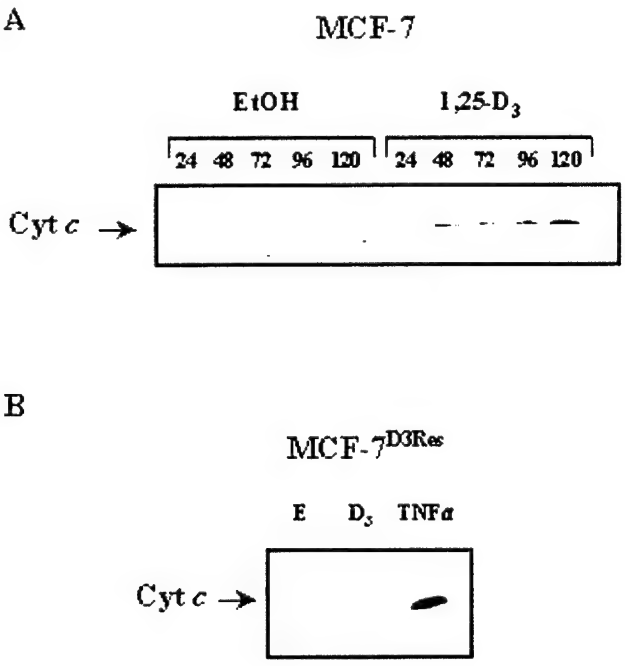

FIG. 2. Cytosolic localization of cytochrome *c* after treatment with 1,25-$(OH)_2D_3$ or TNFα. A. Time course of cytosolic cytochrome *c* after treatment with vehicle control (ethanol) or 100 nM 1,25-$(OH)_2D_3$ in MCF-7 cells. Cells were plated at density of $1x10^5$ cells/150-mm dish. Two days after plating, the cells were treated with ethanol or 1,25-$(OH)_2D_3$ and re-fed two days later. S100 fractions prepared at the indicated time points as described in *Experimental Procedures* were separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with cytochrome *c* (7H8.2C12) antibody. **B.** Cytosolic cytochrome *c* in MCF-7^{D3Res} cells. Cells were plated and treated with ethanol or 1,25-$(OH)_2D_3$ as described above and 2.5 ng/ml TNFα was added 3 days before harvest. All the dishes were harvested on day 5 of treatment, and S100 fractions were prepared and immunoblotted as described above. The results are representative of at least three independent experiments.

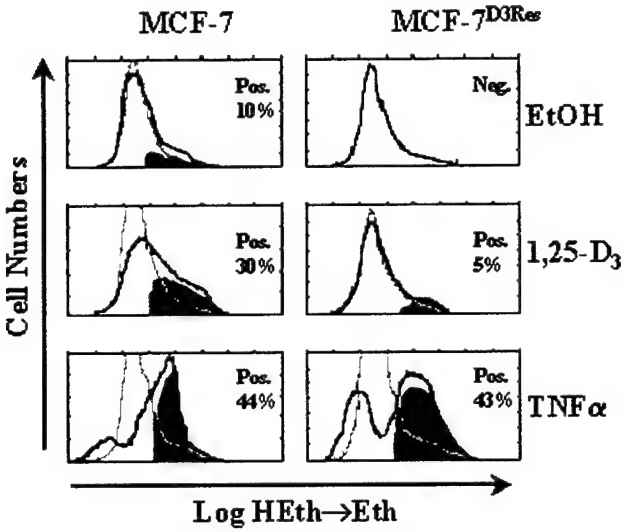

FIG. 3. ROS production after treatment with 1,25-$(OH)_2D_3$ or TNFα in parental MCF-7 or MCF-7^{D3Res} cells. Cells were plated at a density of $1x10^5$ cells/150-mm dish. Two days after plating, the cells were treated with vehicle control (ethanol), 100 nM 1,25-$(OH)_2D_3$, or media only, re-fed two days later, and 2.5 ng/ml TNFα was added to dishes containing media only. All dishes were harvested on day 5 when ROS generation was assessed by flow cytometry as described in *Experimental Procedures*. Data are expressed as the percentage of cells positive for ROS after negative subtraction of data derived from vehicle treated MCF-7^{D3Res} cells. The results are representative of at least three independent experiments.

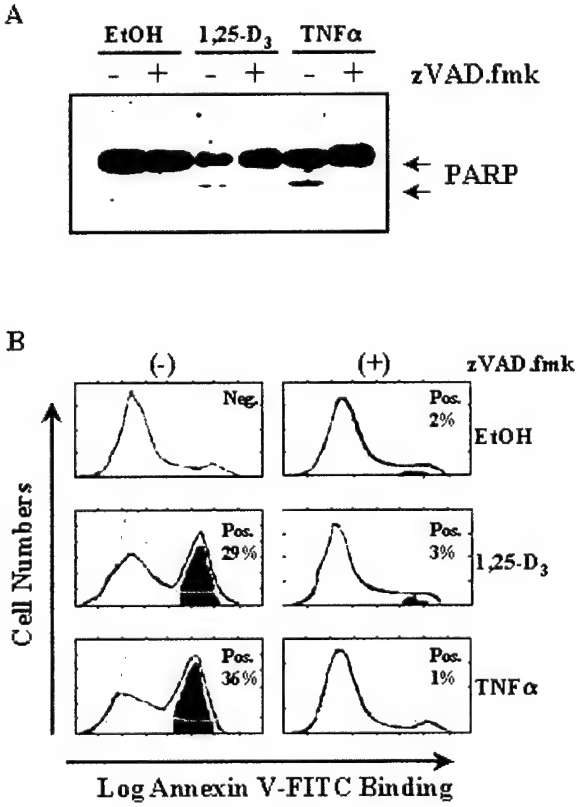

FIG. 4. Effects of caspase inhibitor on PARP cleavage and PS exposure after treatment with 1,25-$(OH)_2D_3$ or TNFα in MCF-7 cells. A. PARP cleavage. Cells were plated and treated as described in FIG. 3 in the presence or absence of 25 μM zVAD.fmk. All the dishes were harvested on Day 5. Nuclear extracts prepared as described in *Experimental Procedures* were separated on SDS-PAGE and immunoblotted with mouse monoclonal antibody to PARP. **B.** PS externalization. Cells plated and treated as described above were incubated with annexin V-FITC and PI as described in *Experimental Procedures*. Data are expressed as the percentage of annexin V-FITC positive cells after negative subtraction of data generated with vehicle treated cells. The results are representative of at least three independent experiments.

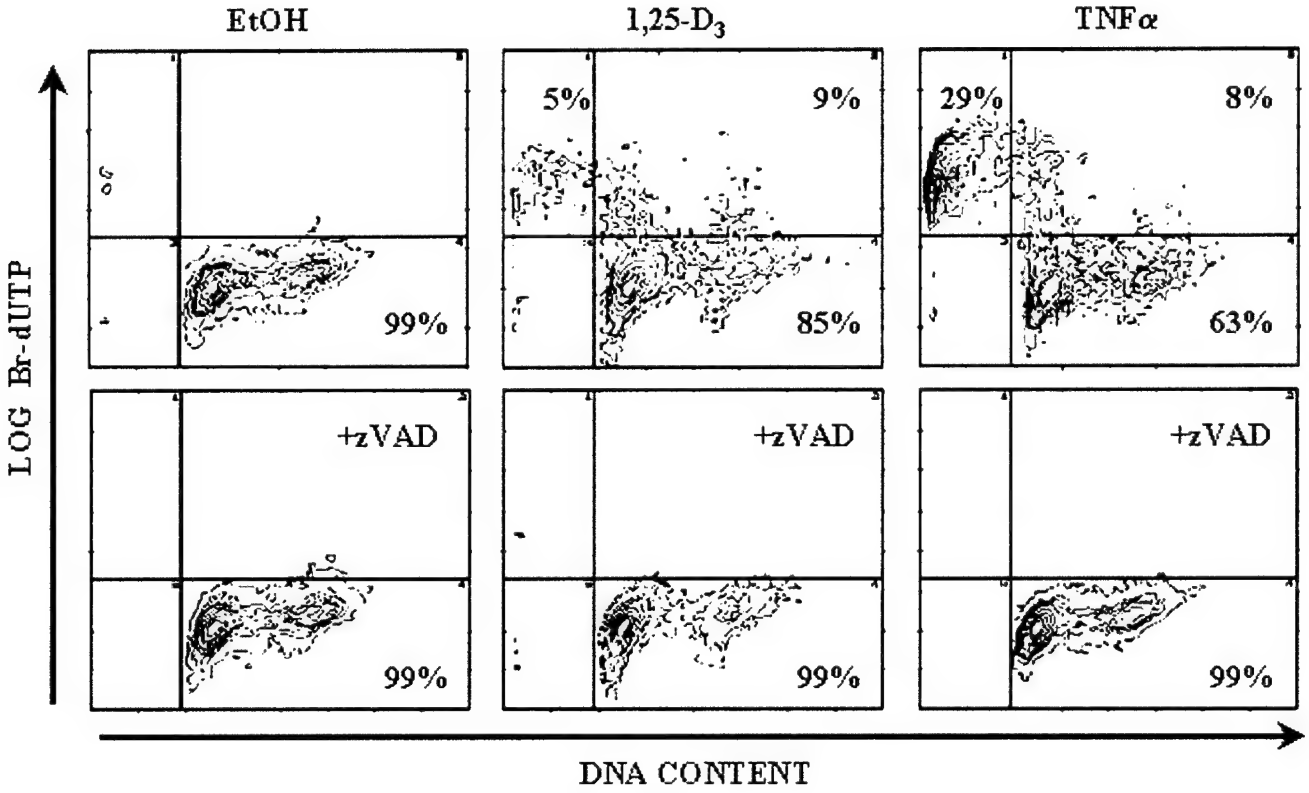

FIG. 5. Effect of caspase inhibitor on DNA fragmentation after treatment with 1,25-$(OH)_2D_3$ or TNFα in MCF-7 cells. Cells were plated and treated as described in FIG. 4, and DNA fragmentation was determined by flow cytometry as described in *Experimental Procedures*. The results are representative of at least three independent experiments.

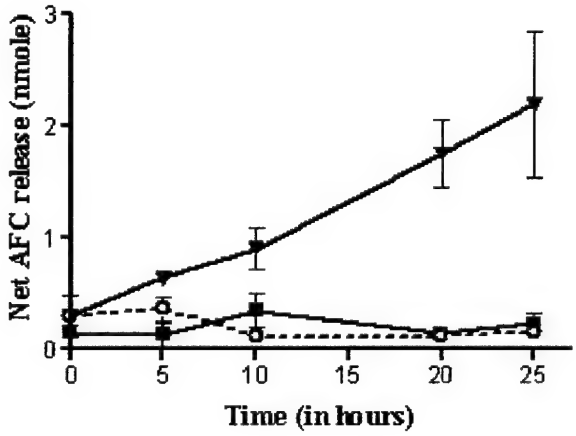

FIG. 6. Time course of DEVDase cleavage activity after treatment with 1,25-$(OH)_2D_3$ or TNFα in MCF-7 cells. Cells were plated at a density of $2x10^5$ cells/150-mm dish. Two days after plating, the cells were pretreated with ethanol, 100 nM 1,25-$(OH)_2D_3$ or media only. Two days later, medium was replaced with ethanol (o), 100 nM 1,25-$(OH)_2D_3$ (■), or 10 ng/ml TNFα (▼) (T=0). Cytosolic extracts of cells harvested at the indicated time points were incubated with DEVD-AFC for 1 h at 37°C, and analyzed on a fluorescence spectrophotometer. Data represent mean ± SEM of two independent experiments performed in duplicate.

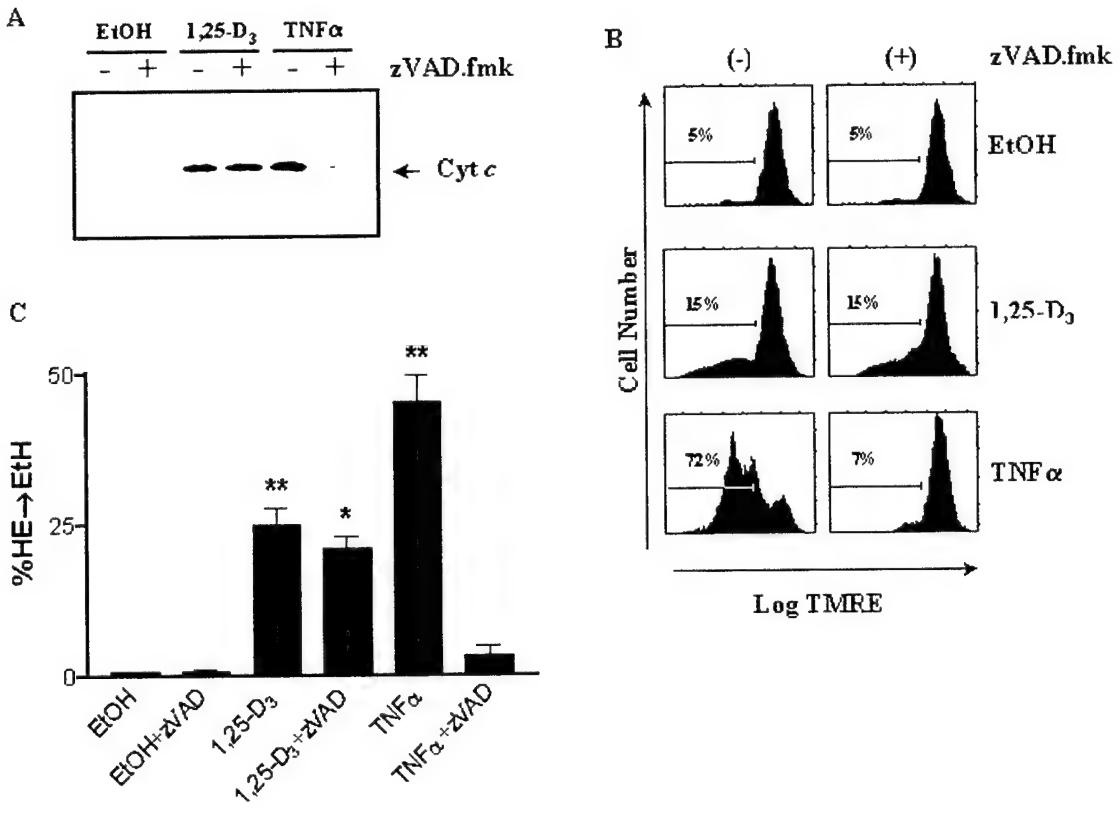

FIG. 7. Effects of caspase inhibitor on cytochrome *c* release, mitochondrial membrane potential, and ROS production after treatment with 1,25-$(OH)_2D_3$ or TNFα in MCF-7 cells. A. Cytochrome *c* release. S100 extracts prepared from cells treated as described in FIG. 4 were separated on SDS-PAGE and immunoblotted with cytochrome *c* (7H8.2C12) mouse monoclonal antibody. **B.** Mitochondrial membrane potential. Cells plated and treated as described above were incubated with 1 μM TMRE as described in *Experimental Procedures* and analyzed by flow cytometry. Data are expressed as the percentage of cells with reduced mitochondrial membrane potential. **C.** ROS production. Cells treated as described above were incubated with 4 μM HE as described in *Experimental Procedures* and analyzed by flow cytometry. The data represent the percentage of cells positive for ROS. Each *bar* represents the mean ± SEM of 2-4 independent experiments. **, $p<0.001$; *, $p<0.01$; treated *vs.* ethanol control as evaluated by ANOVA. The results (**A** & **B**) are representative of at least three independent experiments.

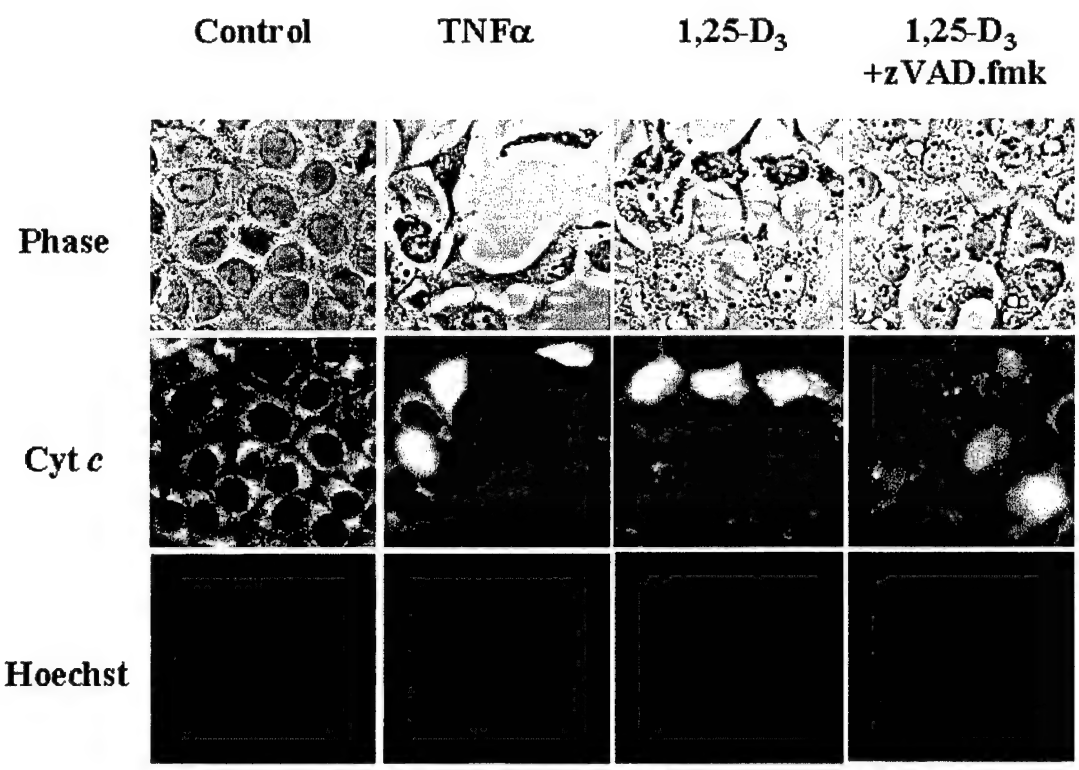

FIG. 8. Effects of 1,25-$(OH)_2D_3$ or TNFα on morphology and cytochrome *c* release in MCF-7 cells. Cells grown on Lab-Tek II chamber slides were treated with ethanol, 100 nM 1,25-$(OH)_2D_3$, or 2.5 ng/ml TNFα ± 25 μM zVAD.fmk, fixed after 96 h (ethanol, 1,25-$(OH)_2D_3$) or 48 h (TNFα), immunostained with cytochrome *c* (6H2.B4) mouse monoclonal antibody and visualized with ALEXA-488 conjugated secondary antibody. Nuclei were counterstained with Hoechst 33258. The images were taken with an Olympus AX70 fluorescence microscope at 400x magnification. *Top,* phase contrast; *Middle,* cytochrome *c* (green); *Bottom,* Hoechst (blue). The results are representative of at least two independent experiments.

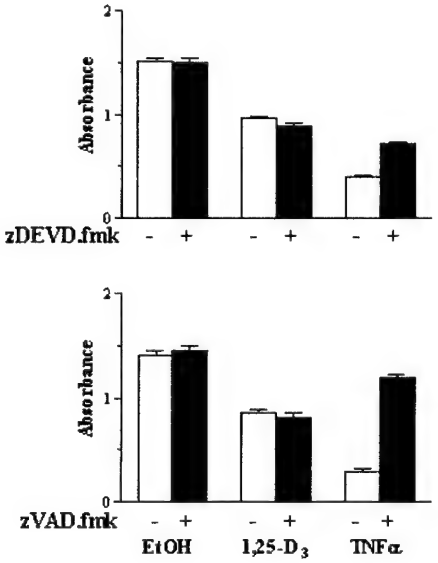

FIG. 9. Effect of caspase inhibitors on MCF-7 cell number after treatment with 1,25-$(OH)_2D_3$ or TNFα. Cells were seeded at a density of 1000 cells/well in 24-well plates. Two days after plating, cells were treated with ethanol, 100 nM 1,25-$(OH)_2D_3$ or 10 ng/ml TNFα for 5 days ± 25 μM zVAD.fmk (broad spectrum, *Bottom*) or zDEVD.fmk (caspase-3/7 specific, *Top*). Total cell number was determined by crystal violet assay as described in *Experimental Procedures.* Data represent mean ± SEM of four replicate determinations. The results are representative of at least two independent experiments.

Title: ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS

Authors: Carmen J. Narvaez, Thomas Waterfall, JoEllen Welsh

Key Words: *breast cancer cells, apoptosis, vitamin D resistance, mitochondria, cytochrome c*

Cell Lines: *MCF-7, MCF-7^{D3Res}*

ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS

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Introduction 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the active form of vitamin D_3, acts through the nuclear vitamin D receptor (VDR) and is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo*. Our lab has shown that 1,25-$(OH)_2D_3$ induces morphological and biochemical markers of apoptosis (chromatin and nuclear matrix condensation, and DNA fragmentation) in MCF-7 breast cancer cells (1). The precise mechanism of how 1,25-$(OH)_2D_3$ and its nuclear receptor, the VDR, mediate apoptosis is poorly understood.

We examined the 1,25-$(OH)_2D_3$ signaling pathway downstream of the VDR in order to identify specific intracellular events involved in 1,25-$(OH)_2D_3$ mediated apoptosis and to characterize events which are blocked in MCF-7^{D3Res} cells (a vitamin D_3-resistant variant)(2). In particular, the effects of 1,25-$(OH)_2D_3$ mediated apoptosis on mitochondrial function and caspase activity were studied and compared to the effects of TNFα. TNFα was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by means of a well-defined pathway triggered by TNFR1, a cell surface death receptor whose signaling results in caspase activation and disruption of mitochondrial function (3). Caspases are a family of evolutionarily conserved cysteine proteases that become activated upon proteolytic cleavage, and are responsible for cell disassembly. Mitochondria play a central role in controlling cell death. Translocation of Bax from cytosol to mitochondria, release of cytochrome *c*, and activation of caspases may initiate disruption of mitochondrial function (4,5). It may be during this mitochondrial phase that the cell makes a commitment to die. Events downstream of mitochondrial disruption are characterized by the action of caspases and nuclease activators released from mitochondria leading to the ultimate destruction of the cell.

While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase dependent pathways. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to determine the involvement of caspase-dependent proteolysis in 1,25-$(OH)_2D_3$ mediated apoptosis.

Results and Discussion Disruption of mitochondrial function is one of the primary events that occur during apoptosis. Translocation of Bax to the mitochondrial outer membrane has been implicated in induction of apoptosis. Bax

redistribution to mitochondria occurs in the presence of both 1,25-$(OH)_2D_3$ and TNFα in MCF-7 cells (Figure 1). Not only was Bax translocated to mitochondria, but it was also cleaved from 21 kDa to 18 kDa. This observation is consistent with other reports of Bax cleavage during drug-induced apoptosis (6). Bax translocation to mitochondria and apoptosis in response to TNFα can be triggered in MCF-7^{D3Res} cells indicating that Bax functions appropriately during apoptosis induced by agents other than 1,25-$(OH)_2D_3$ (Figure 1).

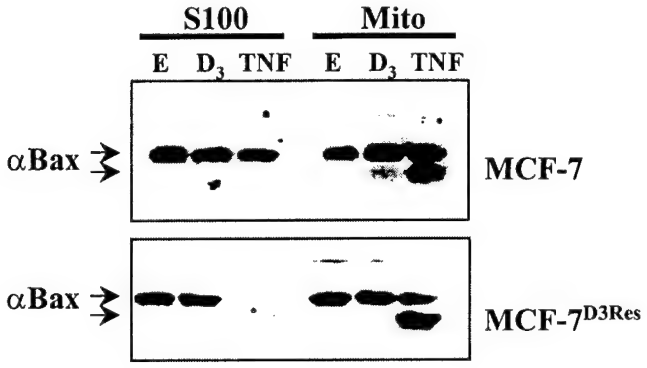

Figure 1 Subcellular distribution of Bax in MCF-7 and MCF-7^{D3Res} cells after treatment with 1,25-D_3 or TNFα.

Translocation of Bax to mitochondria is associated with subsequent release of cytochrome *c*, events that are considered to be commitment points for activating apoptosis. Cytochrome *c* normally resides within the intermembrane space of live cells. Cytochrome *c* is not detected in the cytosolic fraction of vehicle treated control cells. However, 1,25-$(OH)_2D_3$ induces redistribution of cytochrome *c* from mitochondria to cytosol as early as 48 hrs in MCF-7 cells, before any morphological apoptotic events are detected. By contrast, TNFα, but not 1,25-$(OH)_2D_3$, induces release of cytochrome *c* in MCF-7^{D3Res} cells.

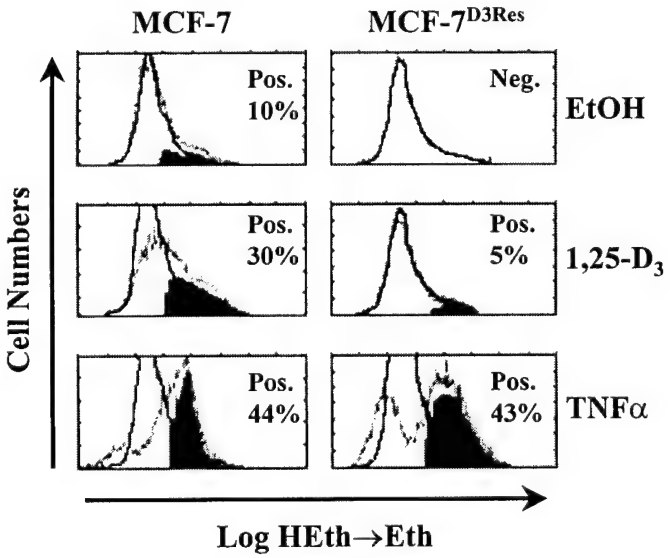

Figure 2 ROS production in MCF-7 or MCF-7^{D3Res} cells after treatment with 1,25-D_3or TNFα.

Long term exclusion of cytochrome *c* from the electron transport chain can lead to impairment of proton flow, and generation of reactive oxygen species (ROS) due to incomplete reduction of molecular oxygen (7). Hence, mitochondrial generation of ROS in response to apoptotic stimuli was examined. By using flow cytometric techniques, production of superoxide anion was assessed by the degree of oxidation of hydroethidine to ethidium, a DNA stain that fluoresces red upon DNA intercalation.

MCF-7 cells, but not MCF-7^{D3Res}, produced ROS in the presence of 1,25-$(OH)_2D_3$, whereas TNFα induced ROS production in both cell lines (Figure 2).

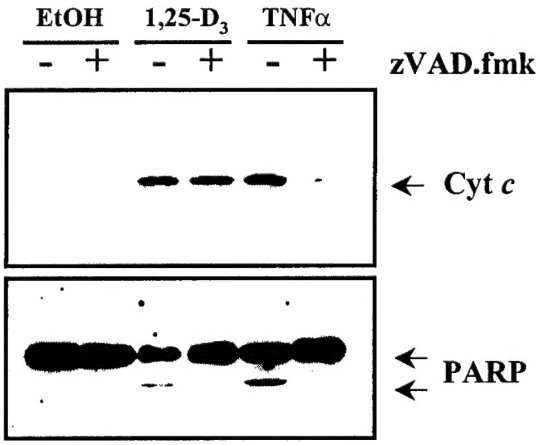

Figure 3 Expression of cytochrome c and PARP in MCF-7 cells (in the presence or absence of zVAD.fmk).

Cytochrome *c* release into the cytosol triggers caspase activity downstream of mitochondria. In order to determine the involvement of caspase-dependent proteolysis in 1,25-$(OH)_2D_3$ mediated apoptosis, we used a broad-spectrum cell permeable inhibitor (zVAD.fmk) in MCF-7 cells. Proteolytic activity associated with caspases was analyzed by three different methods. First, caspase activity was detected by immunoblot analysis of the cleavage of an endogenous caspase substrate, poly (ADP-ribose) polymerase (PARP). PARP was cleaved in the presence of both 1,25-$(OH)_2D_3$ and TNFα. The cleavage was blocked by zVAD.fmk (Figure 3). The second and third methods utilized flow cytometry to analyze phosphatidylserine (PS) exposure and DNA fragmentation, respectively, which others have shown can be provoked by caspases (8). Annexin V-FITC is a convenient probe for monitoring changes in the distribution of PS in the plasma membrane during apoptosis. Both 1,25-$(OH)_2D_3$ and TNFα induced PS exposure in MCF-7 cells, which was blocked by zVAD.fmk treatment. DNA fragmentation was assessed by incorporation of bromodeoxyuridine by terminal transferase and detection by anti-bromodeoxyuridine antibody conjugated to FITC. Both 1,25-$(OH)_2D_3$ and TNFα induced DNA fragmentation, which was completely blocked by zVAD.fmk. However, TNFα, but not 1,25-$(OH)_2D_3$, induced DEVDase (caspase-3/7) cleavage activity in MCF-7 cells. This observation indicates that other, or as yet unidentified, effector caspases may be responsible for 1,25-$(OH)_2D_3$ mediated PARP cleavage, PS exposure, or DNA fragmentation.

Since zVAD.fmk blocked caspase activity downstream of mitochondria, we examined the effects of the caspase inhibitor on cytochrome *c* release and mitochondrial activity. The caspase inhibitor had no effect on 1,25-$(OH)_2D_3$ induced cytochrome *c* release (Figure 3), decrease in mitochondrial membrane potential, or ROS production. However, caspase inhibitor was able to block all mitochondrial events in response to TNFα. This demonstrates that TNFα induced cell death occurs by a caspase-dependent mechanism consistent with a role for caspase-8 in triggering cytochrome *c* release.

Since zVAD.fmk was unable to block cytochrome *c* release and mitochondrial dysfunction in response to 1,25-$(OH)_2D_3$, we determined the effect of caspase inhibitor on cell death and clonogenic potential. Both zVAD.fmk and zDEVD.fmk

caspase inhibitors protected MCF-7 from TNFα cell death with zVAD.fmk exhibiting the greatest response. However, neither caspase inhibitor could protect MCF-7 cells from 1,25-$(OH)_2D_3$ mediated apoptosis since the reduction in cell number was not blocked by these inhibitors. In addition, MCF-7 cells treated with 1,25-$(OH)_2D_3$ lost their clonogenic potential even when they were treated in the presence of zVAD.fmk. This suggests that the activation of caspases by 1,25-$(OH)_2D_3$ occurs subsequent to the events that commits the cells to die.

Conclusion This study demonstrates for the first time that 1,25-$(OH)_2D_3$ induces apoptosis in MCF-7 cells by disrupting mitochondrial function, which is accomplished by translocation of Bax to mitochondria, release of cytochrome *c*, production of ROS, and decrease in mitochondrial membrane potential. This is the first data that demonstrates that 1,25-$(OH)_2D_3$ signaling on mitochondria does not require caspase activation, since broad-spectrum caspase inhibitor zVAD.fmk was unable to block these mitochondrial events. The failure of 1,25-$(OH)_2D_3$ to disrupt mitochondrial function in MCF-7^{D3Res} cells suggests that 1,25-$(OH)_2D_3$ signaling on mitochondria is a complex event requiring more than a functional VDR. Events upstream of Bax translocation to mitochondria in response to 1,25-$(OH)_2D_3$ are abrogated in the vitamin D3-resistant cells, and contributed to resistance to 1,25-$(OH)_2D_3$ mediated apoptosis. Caspases act solely as executioners by facilitating 1,25-$(OH)_2D_3$ mediated apoptosis, but caspase activation is not required for induction of cell death by 1,25-$(OH)_2D_3$ in MCF-7 cells. Although caspase inhibitor blocked all the biochemical changes associated with caspase activation occurring following perturbation of mitochondria and loss of cytochrome *c*, the commitment of MCF-7 cells to 1,25-$(OH)_2D_3$ mediated apoptosis is caspase independent.

References

1. Simboli-Campbell, M., Narvaez, C. J., Tenniswood, M., and Welsh, J. (1996) *J Steroid Biochem Mol Biol* **58,** 367-76
2. Narvaez, C. J., Vanweelden, K., Byrne, I., and Welsh, J. (1996) *Endocrinology* **137,** 400-9
3. Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) *Annu Rev Cell Dev Biol* **15,** 269-90
4. Tsujimoto, Y., and Shimizu, S. (2000) *FEBS Lett* **466,** 6-10
5. Susin, S. A., Zamzami, N., and Kroemer, G. (1998) *Biochim Biophys Acta* **1366,** 151-65
6. Wood, D. E., and Newcomb, E. W. (2000) *Exp Cell Res* **256,** 375-382
7. Cai, J., and Jones, D. P. (1998) *J Biol Chem* **273,** 11401-11404
8. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312-6

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ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS. CJ Narvaez and J Welsh Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556

Vitamin D compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D_3 (1,25-D_3), the active form of vitamin D_3, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-D_3 mediated apoptosis in MCF-7 breast cancer cells *in vitro*. Treatment of MCF-7 cells with 1,25-D_3 induced DNA fragmentation, external display of phosphatidylserine (PS), and cleavage of the caspase substrate, poly (ADP-ribose) polymerase (PARP). Mitochondrial events associated with 1,25-D_3 mediated apoptosis included translocation of Bax, disruption of mitochondrial membrane potential, generation of reactive oxygen species (ROS), and release of cytochrome *c*. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (z-Val-Ala-Asp-fluoromethylketone, zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D_3 mediated apoptosis. The effect of 1,25-D_3 on MCF-7 cells was compared to that of tumor necrosis factor α (TNFα), which induces apoptosis via a caspase-dependent pathway. zVAD.fmk prevented 1,25-D_3 mediated PARP cleavage, PS exposure, and DNA fragmentation. In contrast, zVAD.fmk did not prevent 1,25-D_3 mediated redistribution of Bax from cytosol to mitochondria, loss of mitochondrial membrane potential, generation of ROS, or release of cytochrome *c*. In addition, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-D_3 treatment of a MCF-7 cell clone selected for resistance to 1,25-D_3 mediated apoptosis. Most significantly, zVAD.fmk did not protect MCF-7 cells from 1,25-D_3 induced apoptosis, indicating that the commitment of MCF-7 cells to 1,25-D_3 mediated cell death is caspase independent. The sensitivity of 1,25-D_3 induced apoptosis to caspase inhibition was found to be distinct from that of TNFα induced apoptosis, which was completely prevented by zVAD.fmk. In summary, these data clearly indicate that although caspase inhibition can block some of the late stages of 1,25-D_3 mediated apoptosis in MCF-7 cells; the commitment to cell death is caspase independent. Our data implicate Bax distribution and mitochondrial disruption as critical caspase independent events in 1,25-D_3 mediated apoptosis of breast cancer cells. *Supported by NIH CA69700 & DAMD17-97-1-7183.*

Poster presented at the ***Era of Hope*** **Department of Defense Breast Cancer Research Progam Meeting** in Atlanta, GA, June 8-11, 2000.

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Vitamin D compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D_3 (1,25-D_3), the active form of vitamin D_3, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-D_3 mediated apoptosis in MCF-7 breast cancer cells *in vitro*. Treatment of MCF-7 cells with 1,25-D_3 induced DNA fragmentation, external display of phosphatidylserine (PS), and cleavage of the caspase substrate, poly (ADP-ribose) polymerase (PARP). Mitochondrial events associated with 1,25-D_3 mediated apoptosis included translocation of Bax, disruption of mitochondrial membrane potential, generation of reactive oxygen species (ROS), and release of cytochrome *c*. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (z-Val-Ala-Asp-fluoromethylketone, zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D_3 mediated apoptosis. The effect of 1,25-D_3 on MCF-7 cells was compared to that of tumor necrosis factor α (TNFα), which induces apoptosis via a caspase-dependent pathway. zVAD.fmk prevented 1,25-D_3 mediated PARP cleavage, PS exposure, and DNA fragmentation. In contrast, zVAD.fmk did not prevent 1,25-D_3 mediated redistribution of Bax from cytosol to mitochondria, loss of mitochondrial membrane potential, generation of ROS, or release of cytochrome *c*. In addition, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-D_3 treatment of a MCF-7 cell clone selected for resistance to 1,25-D_3 mediated apoptosis. Most significantly, zVAD.fmk did not protect MCF-7 cells from 1,25-D_3 induced apoptosis, indicating that the commitment of MCF-7 cells to 1,25-D_3 mediated cell death is caspase independent. The sensitivity of 1,25-D_3 induced apoptosis to caspase inhibition was found to be distinct from that of TNFα induced apoptosis, which was completely prevented by zVAD.fmk. In summary, these data clearly indicate that although caspase inhibition can block some of the late stages of 1,25-D_3 mediated apoptosis in MCF-7 cells, the commitment to cell death is caspase independent. Our data implicate Bax distribution and mitochondrial disruption as critical caspase independent events in 1,25-D_3 mediated apoptosis of breast cancer cells.

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